

**Isolation and Identification of *Bacillus cereus* s.l. from Ready-to-eat
Cereals**

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LIST OF ABBREVIATIONS

cfu	colony-forming unit
DNA	deoxyribonucleotide acid
g	gram
H	hour
ml	millilitre
RTE	ready-to-eat
<i>s. l.</i>	<i>sensu lato</i>
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultraviolet
VP	Voger – Proskeuer
δ	delta
°C	degree Celcius
%	percentage
\leq	less than or equal to

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Isolation and Identification of *Bacillus cereus* s.l. from Ready-to-eat Cereals

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ABSTRACT

Bacillus cereus is a soil inhabitant gram positive bacterium, but can also be found in raw or cooked starchy foods, such as the highly-processed ready-to-eat (RTE) foods. In this study, *Bacillus cereus* s.l. was isolated from RTE cereals and identified using biochemical tests. A total of 30 RTE cereals were purchased from the local supermarket in Kuching and Kota Samarahan, Sarawak from December 2009 until February 2010. Samples were enriched in Tryptic Soy Broth for 24 hours. Then, *Bacillus cereus* selective agar (base) was used to isolate the colonies. Isolated presumptive colonies were based on their morphological growth on the agar, which are large, round and pinkish white colonies. After that, Gram stain and a series of biochemical tests such as catalase test, motility, Tyrosine Decompose, Glucose fermentation, VP test and Indole test were carried out. Later, the most highly suspected colonies were subjected to BBL Crystal™ Identification Systems Gram - Positive ID Kit test for further confirmation of the organisms. Result has revealed that four food samples were detected to be contaminated by *Bacillus cereus* s.l.. Therefore, it is very crucial for food industries and health department to pay attention to the safety on consumption of RTE cereals, as it is possible that *Bacillus cereus* s.l. exist in high count number and pose hazardous effects to consumers.

Keywords: *Bacillus cereus* s.l., gram positive, ready-to-eat cereals, isolated, biochemical test

ABSTRAK

Bacillus cereus s.l. adalah bakteria penghuni tanah yang bergram positif, tetapi juga boleh ditemui dalam makanan bertepung sama ada mentah atau dimasak, terutamanya makanan siap-sedia-makan (RTE). Dalam kajian ini, *Bacillus cereus* s.l. dipencilkan dari bijirin RTE dan diidentifikasi dengan menggunakan ujian biokimia. Sebanyak 30 bijirin RTE telah dibeli dari pusat beli-belah di Kuching dan Kota Samarahan, Sarawak dari Disember 2009 hingga Mac 2010. Sampel makanan diperkaya dalam Soy Tryptic Broth selama 24 jam. Kemudian *Bacillus cereus* Selective Agar (base) digunakan untuk mengasingkan koloni bakteria. Koloni yang dipencilkan adalah berdasarkan morfologi pertumbuhan pada agar, iaitu koloni adalah besar, bulat dan berwarna putih-merah muda. Selepas itu, Gram Stain dan siri ujian biokimia seperti katalase, motiliti, pereputan Tirosin (Tyrosine Decompose), Glukosa fermentasi, ujian VP dan Indole dijalankan. Selepas itu, koloni yang paling disyaki dikendalikan ujian menggunakan BBL Crystal™ Identification Systems Gram - Positive ID Kit untuk pengesahan lanjutan bagi *Bacillus cereus* s.l.. Keputusan menunjukkan bahawa empat sampel makanan dikesan telah dicemar oleh *Bacillus cereus* s.l.. Oleh itu, pihak dari industri makanan dan jabatan kesihatan harus memberi perhatian pada keselamatan konsumsi bijirin RTE, kerana jumlah koloni bakteria yang tinggi dapat menimbulkan masalah kesihatan kepada pengguna.

Kata kunci: *Bacillus cereus* s.l., gram positif, bijirin siap-untuk-makan, dipencilkan, ujian biokimia

CHAPTER 1

INTRODUCTION

1.1 Introduction

Bacillus cereus is Gram-positive with rod shape structure and forms spores. The spores are able to survive in hot and dry conditions, and remain dormant for many years (Sagripanti *et al.*, 2006; Henriques and Moran, 2007). *Bacillus cereus* is motile and able to live and grow well in both aerobic as well as anaerobic environments (Granum and Lund, 1997). It is commonly present in soil, but can be found in foods, such as dairy products, rice, cereals and cereals derivatives, dried foods, spices, eggs, vegetables and meats (Kramer and Gilbert, 1989; Granum, 2005).

Based on some researches, the *Bacillus cereus* should be placed under the group of *Bacillus cereus s.l.*. This group comprises the strains of *Bacillus cereus*, *Bacillus anthracis* and *Bacillus thuringiensis*. This is because their pathogenicity and virulence gene can be transferred between each other by plasmid (Gonzales *et al.*, 1982; Sabelnikov and Ulyashova, 1990; Helgason *et al.*, 2000b). For example, *cry* gene of *Bacillus thuringiensis* could be found in *Bacillus cereus* strain (Reddy *et al.*, 2009). Additionally, *Bacillus thuringiensis* strain has been reported to produce enterotoxin and cause the gastroenteritis outbreak (Jackson *et al.*, 1995). The enterotoxin is not typically characteristic for *Bacillus thuringiensis*, but is for *Bacillus cereus*. Moreover, these three strains also share high degree of homology in chromosomal DNA (Helgason *et al.*, 2000b). Therefore, scientists can only differentiate these three strains using molecular approach by studying their plasmid. Without the presence of plasmid, *Bacillus cereus*, *Bacillus anthracis* and *Bacillus thuringiensis* cannot be differentiated (Thorne, 1993). Somehow by biochemical test approach, motility test, haemolytic and tyrosine decomposed ability are the only way to

differentiate *Bacillus cereus* and *Bacillus thuringiensis* from *Bacillus anthracis* (Rhodehamel and Harmon 2001).

Foods with *Bacillus cereus* contamination will usually trigger emetic (vomiting) and diarrheal. This is due to the emetic toxin and enterotoxins (Drobniewski, 1993) produced by the survived bacteria and their spores during foods processing (Sagripanti *et al.*, 2006; Henriques and Moran, 2007). Outbreaks of *Bacillus cereus* food poisoning are very common in Japan, North America, and Europe countries such as Norway, Netherland and Iceland (Kramer and Gilbert, 1989; Griffiths and Schraft, 2002). Due to the low toxicity of *Bacillus cereus* poisoning (Garbutt, 1997) and the ability of most patients to recover within 24 hours, the numbers of outbreak cases being reported are much lower than the actual cases (Garbutt, 1997; Granum 2007).

Ready-to-eat foods especially cereals are produced under controlled and clean processing conditions (Fang *et al.*, 2003) with strict surveillance from the authority (Wei *et al.*, 2006). Processes are almost fully operated by machines (Hoover's Inc., 2010). Therefore, these foods are considered as to be the lowest contamination by microorganisms and safe to be consumed. However, evidences have shown that high level of *Bacillus cereus* could be found from those ready-to-eat foods, such as instant cereals, sandwiches, rice, pasteurized milk, macaroni and cheese (Holmes *et al.*, 1981; Fang *et al.*, 1997; Notermans *et al.*, 1997; Fang *et al.*, 2003).

Ready-to-eat (RTE) cereals are very common and come with different brands and variations, either from local or imported, can be found in Malaysian market. Several researches revealed that there are many advantages for consuming the RTE cereals, such as lowering blood cholesterol level (Johnson *et al.*, 1998), contributing to a more balance diet with higher daily fiber intake, vitamins and minerals intake (Bertrais *et al.*, 2000),

promoting weight loss (Mattes, 2002) and as nutrients supplement (Naghii and Mofid, 2007).

There were many countries reported that food poisoning outbreaks due to RTE cereals had revealed that *Bacillus cereus s.l.* was the main root of disease (Tay *et al.*, 1982; CDC, 1994; Diereck *et al.*, 2005; Reyes *et al.*, 2006). Therefore, it is instead very important to detect and identify the *Bacillus cereus s.l.* in RTE cereals in order to prevent the outbreak of food poisoning in Malaysia.

In this study, sampling was done on RTE cereals purchased from Kuching and Kota Samarahan, Sarawak. The RTE cereals included were raw cereals, pre-mixed cereal drinks and breakfast cereals. A series of biochemical tests such as the Gram Stain, Catalase, Motility, Trypsin Decompose, Glucose fermentation, Voges - Proskeuer test and Indole test were used to identify the *Bacillus cereus s.l.*. Then, further confirmation of the organisms was done by using BBL Crystal™ Identification Systems Gram - Positive ID Kit, Becton - Dickinson, USA. These conventional methods were successfully be demonstrated to detect the *Bacillus cereus s.l.* in RTE cereals. A total of four out of 30 RTE cereals were contaminated by *Bacillus cereus s.l.*. These include three samples of breakfast cereals and one sample of instant oat.

1.2 Objectives

The objectives of this study are as the following:

- 1) To isolate *Bacillus cereus s.l.* in ready-to-eat (RTE) cereals purchased from Kuching and Kota Samarahan, Sarawak, Malaysia.
- 2) To identified *Bacillus cereus s.l.* in RTE cereals by using a series of biochemical tests and further confirmation using BBL Crystal™ Identification Systems Gram - Positive ID Kit, Becton - Dickinson, USA.
- 3) To determine the prevalence of *Bacillus cereus s.l.* in RTE cereals

CHAPTER 2

LITERATURE REVIEW

2.1 *Bacillus cereus s.l.*

Bacillus cereus is Gram-positive with rod shape structure and forms spores. It is motile due to the presence of peritrichous flagella (Varnam and Evans, 1991). The organism is able to live and grows well in both aerobic as well as anaerobic environments (Granum and Lund, 1997).

Members in the *Bacillus cereus* group include *Bacillus cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides* and *Bacillus weihenstephanensis* (Vilas-Baos *et al.*, 2007). Among these, genomics of *Bacillus cereus*, *Bacillus anthracis* and *Bacillus thuringiensis* are too closely related (by comparing their sequences in 16s RNA) that some studies proposed that the three members should be grouped under a single species, namely *Bacillus cereus s.l.* (Daffonchio *et al.*, 2000; Helgason *et al.*, 2000a, 2000b; Bavykin *et al.*, 2004). ‘s.l.’ is Latin words of ‘sensu lato’ and in English means ‘in the wider sense’ or ‘with the board, or general meaning’ (Greuter *et al.*, 2001).

Few studies have revealed that *Bacillus cereus*, *Bacillus anthracis* and *Bacillus thuringiensis* may undergo horizontal transfer of plasmids that caused these three species receiving or donating the virulence plasmid within each other (Gonzales *et al.*, 1982; Sabelnikov and Ulyashova, 1990; Helgason *et al.*, 2000b). There are other studies which stated that actually *Bacillus cereus*, *Bacillus anthracis*, *Bacillus thuringiensis* can be differentiated through their genetic variations (Chang *et al.*, 2003; Radnedge *et al.*, 2003), that is the different genes they carry in plasmid (Helgason *et al.* 2000b). According to

Thorne (1993), *Bacillus cereus* and *Bacillus thuringiensis* cannot be differentiated if the plasmid is absent in these bacteria.

Bacillus cereus and *Bacillus thuringiensis* share few similar characteristics, such as similarities in morphology, living in a same niche (both live in soil), similar requirement for nutrients and have high degree of DNA homology (Fergus *et al.*, 1988). *Bacillus cereus* strain that carry the *cry* gene will be considered as *Bacillus thuringiensis* (Reddy *et al.*, 2009). The only way to differentiate them is the presence of insecticidal coding genes in the plasmid and also the absence of δ –endotoxin crystals from *Bacillus thuringiensis* (Helgason *et al.*, 2000; Turnbull *et al.*, 1990). *Bacillus anthracis* is different from *Bacillus cereus* in such a way that *Bacillus anthracis* is non-motile, absence of hemolytic toxin and the disease they cause are different (Hoffmaster *et al.*, 2004; Siano *et al.*, 2006). *Bacillus cereus* trigger the vomiting and diarrheal, while *Bacillus anthracis* cause anthrax disease and patients will suffer from difficulty in breathing (Hoffmaster *et al.*, 2004).

2.2 Growth conditions

Bacillus cereus is normally inhabited in soil and water environment (Sofos, 2008). However, it can be found in foods, such as dairy products, rice, cereals and cereal derivatives, dried foods, spices, eggs, vegetables and meats (Kramer and Gilbert, 1989; Aksu *et al.*, 2000; Granum, 2005).

Bacillus cereus is able to grow in a wide range of environment due to its endospore - forming characteristic. According to Johnson *et al.* (1983), *Bacillus cereus* is able to germinate at wide range of temperature between 5 °C – 50 °C. They also reported that at 30 °C, the microorganism germinates at fastest rate with optimum generation time of 26 - 57 minutes. The pH range for its growth is between 4.3 and 9.3 and has water activity in

the range between 0.912 - 0.950 for vegetative growth (Johnson *et al.*, 1983; Forsythe, 2000).

Bacillus cereus forms spores when there is an absence of nutrient. Spores are able to resist high heat, dry condition, toxic chemicals, UV radiation, gamma radiation and other extreme environmental conditions. Spores enter a dormant state with the metabolic activity being stopped, but able to survive for many years (Sagripanti *et al.*, 2006; Henriques and Moran, 2007).

Based on a research by Tatsadjieu *et al.* (2007), *Bacillus cereus* was able to survive and germinate to form vegetative cells in an acidic environment, with pH range from 4.5 – 5.2. However, his studies further proved that when the pH was decreased to higher acidic level, *Bacillus cereus* were unable to germinate, but entered a dormant phase and formed spores.

Cross-contamination of *Bacillus cereus* usually happens between soil, water environment and cereals products or dairy products, which further lead to contamination of processed foods. According to Vissers *et al.* (2007), the soil-living *Bacillus cereus* can contaminate the milk through feces, bedding material, soil or grass that is attached on the cow's teats.

The ability of spores to resist high temperature and dry conditions also contribute to cross-contamination of *Bacillus cereus* between the plant origin and their products. The heat- and dry-resistant characteristics allow *Bacillus cereus* to survive in the highly-processed ready-to-eat cereals (Lake *et al.*, 2004), and subsequently germinates when the condition is favored. *Bacillus cereus* could germinate and reached 50 -500 times from its original number of colonies in 6 hours at temperature below 25 °C or in 48 hours at 10 °C (Fang *et al.*, 1997).

Heating of *Bacillus cereus* in an acidic condition is only able to reduce the numbers of bacteria to a safety level that would not trigger poisoning, but would not kill all the bacteria (Tatsadjieu *et al.*, 2007). Soaking of pulses and cereals prior cooking increase the survival rate and enhance germination of spores of *Bacillus cereus*, especially the subsequent cooking that typically would be taken at short time (Blakey and Priest, 1980). According to Melling and Capel (1978), typical boiling of foods for 30 – 40 minutes was unable to destroy the bacteria, as the emetic toxin producing strains are very high heat resistant up to 126 °C.

2.3 *Bacillus cereus* food poisoning

Bacillus cereus causes two types of food poisoning symptoms, which are emetic (vomiting) and diarrheal. The symptoms are often associated with the enterotoxins and emetic toxin produced by the bacteria (Drobniewski, 1993). An experiment conducted by Granum *et al.* (1993) revealed that food poisoning by *Bacillus cereus* was caused by the ingestion of cells or spores, in which later would produce the enterotoxins. A total number of 10^5 - 10^8 cells are enough as an infective dose to cause illnesses (Kramer and Gilbert, 1989; Granum *et al.*, 1993). However, to detect the toxin in *Bacillus cereus*, the bacteria has to reach 10^7 cells / ml in cultures (Kramer and Gilbert, 1989).

The emetic symptoms are often associated with nausea and vomiting. Emetic symptom is known as ‘short-incubation’ disease (Todar, 2008). It usually occurs within 0.5 to 6 hours after the intake of contaminated foods (FDA, 2009). According to Granum (1994), spores of *Bacillus cereus* were able to survive in the processed food and subsequently produce the emetic toxin. Emetic toxin has very high resistant to pH, high heat of 126 °C and the proteolytic activities of pepsin and trypsin (Kramer and Gilbert, 1989).

Reported cases of emetic symptom are often associated with the starchy foods such as mashed potatoes (Jay *et al.*, 2005). This is because emetic *Bacillus cereus* strains are unable to hydrolyse starch (Shinagawa, 1993; Agata *et al.* 1996; Pirttijärvi *et al.*, 1999, 2000), and starchy foods serve as a favorable substrate to support the growth of bacteria and their subsequent toxin production stage (Griffiths and Schraft, 2002).

For diarrheal symptom, patient will suffer from diarrhea, abdominal pain and abdominal cramp (Turnbull, 1996). Diarrheal symptom is usually known as ‘long-incubation’ type of illness and is triggered by diarrheal toxin (FDA, 2009). The diarrheal toxin, or is known as enterotoxins can be destroyed during cooking (Gilbert, 1979). Besides that, these enterotoxins can also be destroyed by the proteolytic enzymes and the low pH condition in stomach. However, spores can survive during acidic digestion, and may germinate in intestine to form vegetative cells, secreting toxin in the gut (Jensen *et al.*, 2003; Swiecicka *et al.*, 2006).

According to Todar (2008), enterotoxins would activate the intestinal adenylate cyclase enzyme and caused intestinal fluid secretion. This usually occurs within 6 - 15 hours. For most cases, patients are able to recover from this food poisoning in less than 24 hours after onset (FDA, 2009). However in some cases, it may take a longer recovery period depend on the patient’s conditions (Todar, 2008).

2.4 *Bacillus cereus* disease outbreaks

Outbreak of *Bacillus cereus* food poisoning is not as common as *Escherichia coli*, *Vibrio species*, and *Salmonella species*. This are mainly due to *Bacillus cereus* poses a moderate to low level of toxicity, and the outbreaks are much localized, where disease only affect small numbers of people (Garbutt, 1997). The low risk of this food borne illness symptoms, short duration of the illness syndromes and the ability of patients to self-recover (usually

less than 24 hours) has contributed to much lesser of actual food poisoning cases being reported in a country (Garbutt, 1997; Granum 2007), often making it underestimated.

In North America, Europe and Japan, the reported cases of *Bacillus cereus* food poisoning is about 1 % - 22 %, covering 0.7 % - 33 % of the overall reported food borne outbreaks (Griffiths and Schraft, 2002). Japan has the highest cases of the emetic symptom being reported, which is about 10 times frequent than diarrheal symptoms. In Europe, however, diarrheal symptom is reported more often (Granum, 2005). According to Granum (2007), differences in food and cooking traditions among these areas were believed to cause the symptoms occurred so vary.

Endospore forming characteristic in *Bacillus cereus* allows the bacteria to survive at high temperature during food processing such as cooking and pasteurisation (Notermans *et al.*, 1997). When cooked food is stored improperly, such as refrigerated at 10 °C and above (Claus and Berkeley, 1986), the surviving bacteria can germinate and vegetatively growth can occur again. The bacteria can grow to a certain density that can trigger food poisoning (Granum, 2005). Insufficient heating of refrigerated foods and poor food handling habits among humans also contribute to this food borne illness (Chang, 2002). Once the dried foods that are contaminated with the spores are contacted with water, the spores will rehydrate, cell wall break, and finally germination begin (Cronin and Wilkinson 2007; Henriques and Moran, 2007).

According to Tay *et al.* (1982), Singapore reported the first *Bacillus cereus* food poisoning outbreak in year 1971. The outbreak occurred in a military camp and 19 out of 168 armies were infected. Investigation was carried out and revealed that the outbreak was due to improper handling of cooked rice. A similar case happened in the U.S. on July 21, 1993. The outbreak occurred in a Child Day Care Center. A report by CDC (1994) stated

that two staffs and 12 children at the age between 2.5 – 5 years old were infected. The patients suffered from nausea, abdominal cramp and diarrheal. The symptoms took 1.5 – 3.5 hour after consuming the contaminated food, and the patients recovered at range time 1.5 – 22 hours after onset (CDC, 1994).

In 1990, Thailand reported an outbreak of *Bacillus cereus* food poisoning in a sports-day event. The outbreak attacked 485 peoples, and the symptoms that were commonly suffered by the patients were nausea, vomiting, abdominal pain and diarrheal. Laboratory examination uncovered that among the foods available on the sports-day event, the éclairs was highly suspected as the main root of illness. Reasons were the food was prepared in one day earlier and were not properly refrigerated before it was served, hence triggering the growth of *Bacillus cereus* to a level of pathogenicity (Thaikruea *et al.*, 1995).

In 2003, a seven-year-old girl consumed a pasta salad and later suffered from vomiting and respiratory distress. She was sent to hospital and died within 20 minutes. The pasta salad was detected to contain high count number of *Bacillus cereus* (10^7 - 10^8 CFU/g) (Dierick *et al.*, 2005).

In 2004, there was an outbreak of *Bacillus cereus* gastroenteritis in a Stockade Facility, West Palm Beach, Florida. Investigation proved that the cold baked beans and turkey bologna were the two main foods that cause the food poisoning by *Bacillus cereus*. The report revealed that the insufficient cooking of turkey, cross-contamination of raw food materials, and improper temperature storage of food before served had contributed to the outbreak (Florida Department of Health, 2004).

In May 2006, another outbreak was reported from a restaurant located in Hardin County, Kentucky, U. S.. Food samples of steamed rice, chopped vegetables and uncooked

chicken were tested and found to be contained *Bacillus cereus* with high numbers of colony forming units (CFU) / gram of samples. Customers who had consumed the fried rice were 100 % being attacked by the illness. Investigation found that rice cooked during lunch was stored at improper temperatures and then served during dinner (Indukuri, 2006).

In 2006, Reyes *et al.* (2006) did a research on milk products used by the Chilean School Feeding Program. He found out that approximately 46 % of the samples (milk with rice, milk substitute, milk powder, milk-cereal-rice, pudding milk, flan, and mousse) contained *Bacillus cereus*. High counts number (3.0 to 10^4 spores / g) of bacteria was discovered in milk products that contained whole rice, cereals and pulses extruded, and food additives (Reyes *et al.*, 2006).

In 2008, *Bacillus cereus* food poisoning had killed an 81 years old man, and a lady was suffered from vomiting within 12 hours, after they had dined in a restaurant located at Pymble, New South Wales, Australia. Investigation had discovered that the main root for this tragedy was due to the contamination of cream asparagus sauce. According to the investigation report, the cream asparagus sauce had been heated and cooled for several times over a period of more than 48 hours, and the *Bacillus cereus* was in 9.8 million parts when the food was served to the customer (Kennedy, 2008).

There were not many *Bacillus cereus* food poisoning outbreaks being reported in Malaysia. In year 1984, there was an outbreak of *Bacillus cereus* food poisoning was reported in a school hostel located at Klang. There were about 114 students, after consumed the fried noodles, experienced the typical *Bacillus cereus* food poisoning symptoms such as abdominal pain, nausea, vomiting and giddiness. The fried noodles was examined and enumerated that the food was contaminated by 2.3×10^6 of *Bacillus cereus* in every gram of fried noodle. This was the ever first outbreak of *Bacillus cereus* food

poisoning reported in Malaysia (Rampal, 1984). According to the Sarawak State Health Department (2007), there were total of 35 food poisoning cases in local schools and only two cases whereby the *Bacillus cereus* was the root of disease.

2.5 Prevention for food poisoning outbreaks

Inappropriate cooking, storage, chilling practices as well as the holding foods at wrong temperature are the major factors that trigger incidents of foodborne illness. Feijoo *et al.* (1997) reported that at temperature of 32 °C and 23 °C, the generation times for spores and vegetative cells of *Bacillus cereus* in dairy products for coffee are within the range 0.887 to 2.876 hours. In addition to their study, they predicted that at temperature of 32 °C, the *Bacillus cereus* would take 7 – 9 hours to reach the level of capable to cause foodborne illness, which is at 1×10^5 cells / ml of sample. However, at general room temperature (23 °C), a longer holding period, which is 7 - 12 hours is required. Besides that, Shehata and Collins (1971) reported that when the *Bacillus cereus* was at 1×10^5 cfu / ml, the doubling time was 5 – 7 hours in order to reach 1×10^7 cfu / ml at 7.2 °C. In addition, a study on *Bacillus cereus* spores germination has been carried out by Sutherland (1993) and showed that spores at initial inoculum of 1×10^3 spores / ml, would germinate and replicate to the level of 2×10^7 cfu / ml after incubation for 24 hours at 21° C.

According to Briley *et al.* (2001), it is a very common status that *Bacillus cereus* and its spores present in raw dried grains such as rice and cereals. Therefore, by understanding the capability of *Bacillus cereus s.l.* to germinate and generate at wide range of temperature, until they reach a food poisoning level, prevention steps from beginning of foods preparation to foods storage can be engaged (Hobbs and Roberts, 1993).

Schneider *et al.* (2004) explained that during cooking, almost all the possible bacteria would be destroyed, leaving only those heat resistant spores, including the *Bacillus cereus* spores. The spores, without any competition could easily survive and proliferate in foods (Brown, 2000). Schneider *et al.* (2004) also stated that gradually cooling followed by reheating of foods in an alkaline environment could increase the heat resistance of the spores. According to Brown (2000), temperature at 56 °C can destroy the heat sensitive *Bacillus cereus* enterotoxin in 5 minutes, but not the heat stable emetic toxin. As a result, the emetic toxin would later be expressed from the *Bacillus cereus* vegetative cells during the stationary stage in growth phase (ICMSF, 1996).

The guidelines provided by Schneider *et al.* (2004), which are adopted from National Institutes of Health (NIH), the National Institute of Allergy and Infectious Diseases (NIAID), and the National Food Processors Association (NFPA) suggested that general domestic cooking practices such as roasting, frying or pressured steaming can destroy the vegetative cells and spores of *Bacillus cereus*. Besides that, the enterotoxin and emetic toxin can be destroyed by heating the foods at 56 °C for 5 minutes and 126 °C for more than 90 minutes respectively. To prevent spores formation, hot foods should always be maintained at high temperature (60 °C), while cold foods should below 4 °C, without any holding period at room temperature. The maximum holding period of cooked foods at room temperature is 1 hour (Lake *et al.*, 2004). Moreover, cooked foods should be chilled rapidly if not consumed on spot. In any circumstances that foods have to be reheated, the overall food internal temperature should reach 74 °C (Schneider *et al.* 2004). To prevent any cross – contamination, all the kitchen surfaces, cooking equipments and utensils should be thoroughly cleaned, and the leftover, especially cereal dust should be removed from the preparation areas (Hobbs and Roberts, 1993).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Collection

A total of 30 commercial cereal products such as raw cereals, pre-mixed cereal drinks and breakfast cereals were purchased from local supermarkets in Kuching and Kota Samarahan, Sarawak. Then, the samples were transported to the laboratory for analysis.

3.2 Enrichment

Twenty gram of the food sample were weighed under sterile condition, homogenized by a Stomacher in 180 ml pre-enrichment medium (Tryptic Soy Broth). Then, samples were enriched in the Tryptic Soy broth for 20 hours at 35 °C.

3.3 Isolation of *Bacillus cereus* s.l.

A three millimeter loopful of 20 hours culture was transferred from broth and was streaked on *Bacillus cereus* Selective Agar (Base). After that, plates were incubated for 24 hours at 35 °C.

3.4 Confirmation of *Bacillus cereus* s.l.

Three to five presumptive pinkish white colonies on eosin pink medium were picked and transferred to Trypticase Soy Agar (TSA) plates. The TSA plates were incubated overnight at 35 °C. Gram stained and a series of biochemical tests were carried out to identify the isolated bacteria. Then, a commercial kit, BBL Crystal™ Identification Systems Gram-Positive ID Kit, Becton - Dickinson, USA was used on selected highly presumptive of *Bacillus cereus* isolates to further confirm for the identity.

The biochemical tests listed below were based on FDA Bacteriological Analytical Manual (Rhodehamel and Harmon 2001).

3.4.1 Catalase test

A single colony was placed on a drop of three percent (3 %) hydrogen peroxide on a sterilized glass slide. Result is positive if bubbling occurs.

3.4.2 Motility test

A single colony was stabbed down into the centre of semi - solid motility agar and incubated for 24 hours at 35 °C. The type of growth along the stabbed line was examined. Test is positive if growth was radiated out from the central stabbed line, which indicated that the organism is motile.

3.4.3 Glucose fermentation test

A single colony was inoculated into the Phenol red glucose broth and incubated for 24 hours at 35 °C. Tube was shaken vigorously and color of broth was observed. Test is positive for *Bacillus cereus s.l.* if broth colour changed from red to yellow, which indicated that acid has been produced from glucose.

3.4.4 Voges - Proskauer test

A single colony was inoculated into the broth and incubated for 48 hours at 35 °C. Test for production of acetylmethyl-carbinol by adding 15 drops of alpha-naphthol solution and five drops of 40 % potassium hydroxide. Then, tube was shaken and results were observed after holding for one hour at room temperature. Test is positive if pink or red colour was developed.